Cryptic physiological trophic support of motoneurons by LIF revealed by double gene targeting of CNTF and LIF

M. Sendtner*†, R. Götz*†, B. Holtmann*†, J-L. Escary‡, Y. Masu†, P. Carroll†, E. Wolf§, G. Brem§, P. Brûlet‡ and H. Thoenen†

Background: The survival and differentiation of motoneurons during embryonic development, and the maintenance of their function in the postnatal phase, are regulated by a great variety of neurotrophic molecules which mediate their effects through different receptor systems. The multifactorial support of motoneurons represents a system of high security, because the inactivation of individual ligands has either no detectable, or relatively small, atrophic or degenerative effect on motoneurons.

Results: Leukaemia inhibitory factor (LIF) has been demonstrated to support motoneuron survival *in vitro* and *in vivo* under different experimental conditions. However, when LIF was inactivated by gene targeting, there were no apparent changes in the number and structure of motoneurons and no impairment of their function. The slowly appearing, relatively mild degenerating effects in motoneurons that resulted from ciliary neurotrophic factor (CNTF) gene targeting were substantially potentiated by simultaneous inactivation of the LIF gene, however. Thus, in mice deficient in LIF and CNTF, the degenerative changes in motoneurons were more extensive and appeared earlier. These changes were also functionally reflected by a marked reduction in grip strength.

Conclusions: Degenerative disorders of the nervous system, in particular those of motoneurons, may be based on multifactorial inherited and/or acquired defects which individually do not result in degenerative disorders, but which become apparent when additional (cryptic) inherited disturbances or sub-threshold concentrations of noxious factors come into play. Accordingly, the inherited inactivation of the CNTF gene in a high proportion of the Japanese population may represent a predisposing factor for degenerative disorders of motoneurons.

Addresses: *Department of Neurology, University of Würzburg, Josef-Schneider Str. 11, 97080 Würzburg, Germany. †Department of Neurochemistry, Max-Planck-Institute for Psychiatry, Am Klopferspitz 18a, 82152 Martinsried, Germany. ‡Unité d' Embryologie Moléculaire de l´Institut Pasteur, URA 1148 du CNRS, 75724 Paris Cedex 15, France. §Abteilung für Molekulare Tierzucht, Ludwig-Maximilians-Universität München, 80539 München, Germany.

Correspondence to: Hans Thoenen

Received: 20 February 1996
Revised: 12 April 1996
Accepted: 7 May 1996

Current Biology 1996, Vol 6 No 6:686–694

Background

There is rapidly accumulating evidence that the regulation of motoneuron survival during embryonic development, and the maintenance of their function in adulthood, are mediated by a great variety of different molecules. These molecules are produced by the motoneuron target tissues, skeletal muscle, the Schwann cells which ensheath the motoneurons (axons), and possibly by orthograde trophic input from sensory and/or supraspinal neurons [1–4].

Evaluation of the relative importance of the different neurotrophic factors is the object of intense research using antibodies, predominantly in chicks, and using transgenic approaches, such as gene targeting and tissue-specific overexpression in mice. Here, we report on double-knockout experiments, in which the genes encoding ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor LIF) have been inactivated. The results of the experiments reveal that LIF has a cryptic physiological neurotrophic function which is not apparent in mice defective in CNTF alone. This aspect is of particular interest in view of the recent observation that a substantial proportion of the Japanese population have a genetically determined CNTF gene inactivation, which alone (not unexpectedly) does not result in any clinical manifestations [5]. However, these individuals might become more sensitive to motoneuron degeneration by other damaging factors which by themselves would not lead to degenerative changes in motoneurons. The CNTF/LIF double-knockout experiments represent a model for such a situation.

CNTF and LIF, which are members of a family of cytokines, have no significant sequence homology but exhibit similarities in their tertiary structure, which contains four amphipathic helices [6–8]. This family includes molecules that were identified by a variety of biological activities (for review see [9]), such as induction of acute-phase responses and B-cell stimulation (interleukin-6 (IL-6) and IL-11) [10], cytostatic effects on tumour cells (LIF and oncostatin M/OSM), inhibition of embryonic stem cell differentiation (LIF and CNTF), neuronal

survival effects (CNTF), and induction of cardiac myocyte hypertrophy (cardiotrophin-1) [11]. Further analyses revealed that these factors share a variety of functions, such as the support of motoneuron survival (CNTF and LIF) [12,13], the induction of cholinergic differentiation of sympathetic precursor cells (CNTF, LIF and OSM; summarized in [14]) and initiation of acute-phase responses in hepatic cells [9,10,15,16].

Apparently, these effects occur by activation of common cellular receptor components. The effects of CNTF, LIF [17], and most probably OSM [18], are mediated by a heterodimer composed of gp130, the signal-transducing component of the IL-6 receptor, and LIFRβ, which was first identified as an essential signal-transducing component of the LIF receptor [19]. These transmembrane molecules share significant sequence homology, particularly in the cytosolic domain [19], and form a low-affinity LIF and CNTF receptor capable of initiating signal transduction after ligand binding [20–22]. The physiological high-affinity receptors for these ligands require at least one additional component [20,23]. The additional component(s) that convert the low-affinity LIF receptor into a high-affinity receptor are not yet known. In contrast, the high-affinity binding of CNTF to its receptor is known to be mediated by an extracellular CNTF-binding protein, CNTFRα, which is expressed in neurons and skeletal muscle cells [24]. This receptor component lacks a transmembrane domain and is linked by a glycosyl phosphatidyl inositol anchor to the external lipid layer of the cell membrane; but CNTFRα has also been found in soluble form at substantial concentrations in the circulation and in the cerebrospinal fluid [21].

CNTF is synthesized and located (at high concentrations) in the cytosol of myelinating Schwann cells [25,26] and subpopulations of astrocytes within the brain and spinal cord. In contrast to LIF, CNTF lacks a hydrophobic leader sequence, so it is not predicted to be released by the classical secretory pathway from synthesizing cells. Thus, the release of CNTF from Schwann cells after nerve lesion is one possible means by which this molecule can exert a function under pathophysiological conditions. Indeed, CNTF immunoreactivity is detectable in the extracellular space after lesion of the sciatic nerve of adult rats [25]. Moreover, damage to the membrane of Schwann cells could also occur after microtraumata, resulting in permeability of the plasma membrane to proteins [27].

LIF mRNA is expressed at very low levels in the Schwann cells of peripheral nerves. However, after nerve lesion, a rapid and dramatic increase in LIF mRNA expression occurs in the distal and proximal nerve stump in close proximity to the lesion site [28]. This indicates that both LIF and CNTF might become available to motor axons after nerve lesion. In addition, retrograde transport of LIF and CNTF, which seems to occur at very low levels in peripheral nerves of adult rats, increases significantly after peripheral nerve lesion [28,29].

Both CNTF [30] and LIF [31,32] are capable of rescuing facial motoneurons from cell death after axotomy in newborn rats. This indicates that both CNTF and LIF have a protective effect on motoneurons, at least after nerve lesion. In order to determine the physiological role of these two cytokines for motoneurons, we have analyzed mice lacking either CNTF or LIF, or lacking both factors, for deficits in the motor system. Our data indicate that both factors contribute to postnatal maintenance of motoneurons. In addition, in adult mice lacking both CNTF and LIF, the loss of motoneurons after nerve lesion is greater than in mice deficient in CNTF alone. These results indicate that, in the case of CNTF-deficiency, the presence of LIF in the peripheral nervous system prevents severe losses of neurons and impaired neuronal function. These results may also have important consequences for humans, because CNTF deficiency has been reported to occur in at least 1–2 % of the Japanese [5] and European populations (R. Giess and M. Sendtner, unpublished observations).

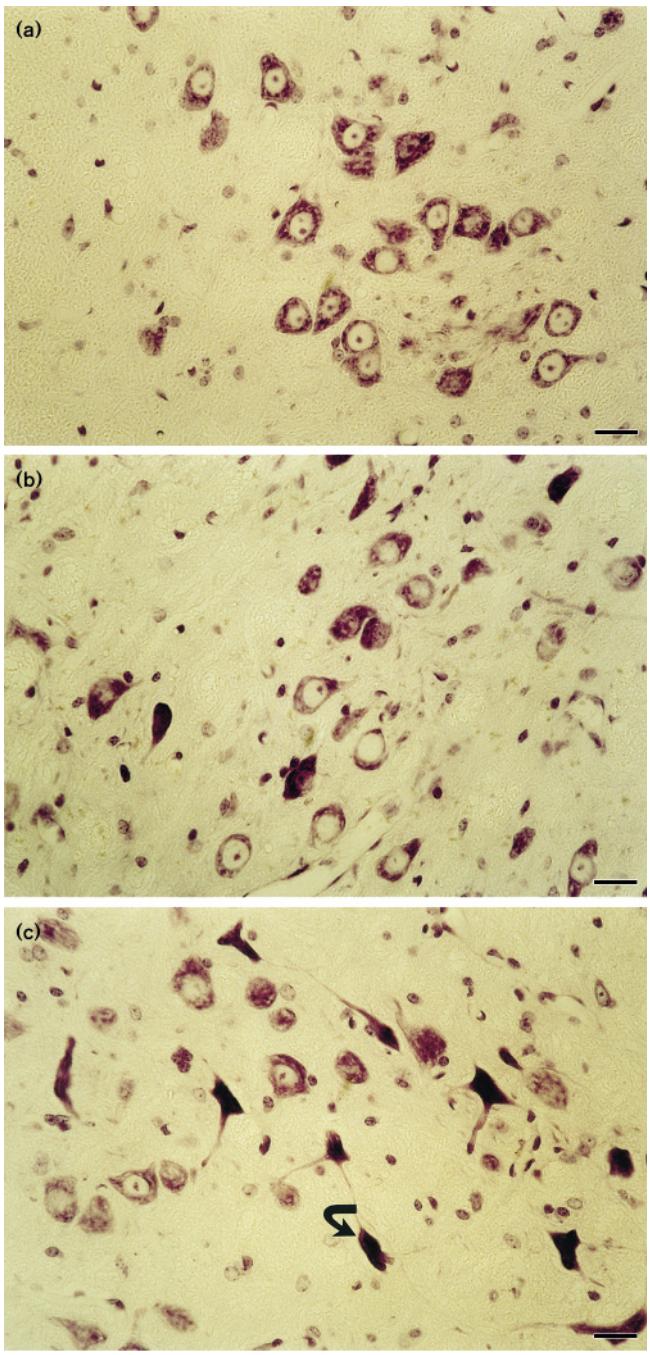
Results

Inactivation of LIF gene expression does not lead to degeneration of motoneurons

In order to compare CNTF and LIF knockout mice with the same genetic background, we crossbred $CNTF^{+/-}$ (129/SV × C57/BL6) mice with $LIF^{+/-}$ mice (129/SV × (c57Bl6 × DBA/2)) and investigated animals from the F2 generation which were either $LIF^{-/-}/CNTF^{+/+}$ or $LIF^{+/+}/CNTF^{-/-}$. These mice were compared with control $LIF^{+/+}/CNTF^{+/+}$ mice.

In agreement with results reported recently [33], $LIF^{-/-}$ mice on this mixed genetic background did not show any abnormalities of motor function or other behavioural changes which could be attributed to defects of the nervous system. In order to identify possible morphological changes that were not sufficient to result in alterations of motor behaviour in $LIF^{-/-}$ mice, we examined facial motoneurons in paraffin sections from 4–8-week-old and 6–9-month-old $LIF^{-/-}$ mice. The facial nucleus was chosen because it lacks interneurons, thus allowing an accurate determination of motoneuron cell numbers by morphological criteria. In contrast to mice lacking functional CNTF expression, we did not observe any pathological alterations in the LIF-deficient animals (Fig. 1a) at the age of 9 months. $CNTF^{-/-}$ mice of the same age showed degenerative changes, such as reduction of the Nissl structure and atrophy of dendritic processes (Fig. 1b). The motoneurons appeared smaller, consistent with previous observations [34]. Serial sections from the brain stems of 6-week-old, 6-month-old and 9-month-old

Figure 1



Morphology of motoneurons in 6- to 9-month-old LIF$^{-/-}$, CNTF$^{-/-}$ and LIF$^{-/-}$/CNTF$^{-/-}$ mice. (a) Facial motoneurons of a 9-month-old LIF$^{-/-}$ mouse. (b) Facial motoneurons of a 6-month-old CNTF$^{-/-}$ mouse. (c) Facial motoneurons of a 6-month-old CNTF$^{-/-}$/LIF$^{-/-}$ mouse. Motoneurons in 6-month-old CNTF$^{-/-}$ mice show a reduction of the Nissl structure and an increase in the ratio between the nuclear and cytoplasmic areas as described [34]. A significant number of motoneurons in which Nissl structure was completely abolished are detectable in CNTF$^{-/-}$/LIF$^{-/-}$ mice. The nuclei of these cells appear severely deformed and are located at an eccentric position very close to the cellular membrane (arrow). In contrast, motoneurons in LIF-deficient mice appear healthy and do not exhibit detectable signs of chromatolysis or other degenerative changes. Scale bar: 25 μm.

animals were prepared and the number of facial motoneurons was determined. In contrast to CNTF$^{-/-}$ mice which showed a 15 % reduction in the number of facial motoneurons between postnatal week 4 and 6 months, there was no reduction in facial motoneuron cell numbers in LIF$^{-/-}$ mice at any age investigated (Table 1).

Inactivation of both the CNTF and LIF genes leads to a significant reduction of motoneurons

Morphological analysis of CNTF$^{-/-}$/LIF$^{-/-}$ mice at 6 months revealed significant pathological changes which were comparable to those observed in CNTF$^{-/-}$ mice (Fig. 1c). Many motoneurons were severely atrophic and showed complete loss of the Nissl structure; in addition, their nuclei were relocated to sites close to the cell membrane (see arrow in Fig. 1c). Such morphological changes were already detectable in CNTF$^{-/-}$/LIF$^{-/-}$ mice at 4–8 weeks (Fig. 2). Determination of facial motoneuron numbers revealed that the number of motoneurons was already substantially reduced in the double-knockout mice at this time point; in contrast, CNTF$^{-/-}$ mice did not yet exhibit any significant loss of motoneurons (Table 1). In order to distinguish whether this reduction in motoneuron number was due to enhanced loss of motoneurons during postnatal development, or to reduced generation during embryonic

Table 1

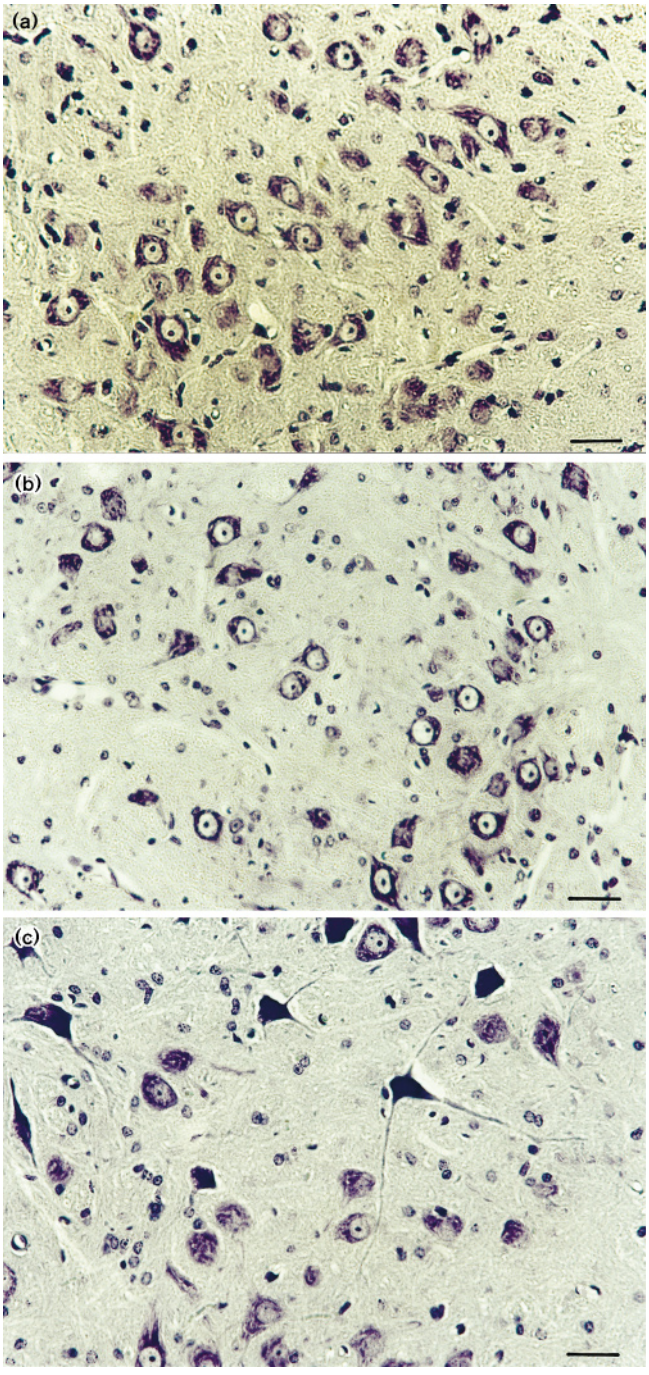
Number of facial motoneurons.

Genotype	Age	Number of facial motoneurons		
Wild-type	1 day	2397 ± 57	$n = 4$*	
CNTF$^{-/-}$/LIF$^{-/-}$	1 day	2420 ± 96	$n = 6$*	n.s.
Wild-type	4–8 weeks	2031 ± 132	$n = 8^{\dagger}$	
CNTF$^{-/-}$	4–8 weeks	2013 ± 135	$n = 7^{\dagger}$	
CNTF$^{-/-}$/LIF$^{-/-}$	4–8 weeks	1604 ± 77	$n = 9^{\dagger}$	$p < 0.05$
Wild-type	6 months	2128 ± 122	$n = 6^{\ddagger}$	
CNTF$^{-/-}$	6 months	1713 ± 104	$n = 4^{\ddagger}$	
LIF$^{-/-}$	6–12 months	2059 ± 71	$n = 5^{\ddagger}$	
CNTF$^{-/-}$/LIF$^{-/-}$	6 months	1575 ± 54	$n = 7^{\ddagger}$	$p < 0.05$

Values shown are mean ± SEM of facial motoneuron counts corrected for split nuclei as described [34]. Statistical significance was tested by Student's *t*-test and one-way ANOVA; the significance of the differences (*p* values) between wild-type and CNTF$^{-/-}$/LIF$^{-/-}$ mice is shown in the last column. *The difference between 1-day-old wild-type and CNTF$^{-/-}$/LIF$^{-/-}$ animals was not statistically significant (n.s.). †The differences between the three groups of mice at 4–8 weeks were tested by one-way ANOVA: at the $p < 0.05$ levels, differences were significant. Both the student's *t*-test and Dunnet's multiple comparison test showed a significant difference between wild-type and CNTF$^{-/-}$/LIF$^{-/-}$ mice, and between CNTF$^{-/-}$ and CNTF$^{-/-}$/LIF$^{-/-}$ mice, but not between wild-type and CNTF$^{-/-}$ mice. ‡The differences between the four groups of mice at 6–12 months were analyzed by one-way ANOVA and found significant at the $p < 0.005$ level. Comparison of individual groups either by Student's *t*-test or Dunnet's multiple comparison test gave the following results: wild-type versus CNTF$^{-/-}$, $p < 0.05$; wild-type versus LIF$^{-/-}$, n.s.; wild-type versus CNTF$^{-/-}$/LIF$^{-/-}$, $p < 0.05$.

development, we determined the number of facial motoneurons in newborn CNTF$^{-/-}$/LIF$^{-/-}$ mice. We found no reduction in the number of facial motoneurons in 1-day-old CNTF$^{-/-}$/LIF$^{-/-}$ mice compared to wild-type mice (Table 1), indicating that the loss of motoneurons seen in the CNTF$^{-/-}$/LIF$^{-/-}$ mice occurs exclusively during postnatal development.

Figure 2



Morphology of facial motoneurons in 6-week-old control, CNTF$^{-/-}$ and CNTF$^{-/-}$/LIF$^{-/-}$ mice. (a) Facial motoneurons of a 6-week-old control mouse. (b) Facial motoneurons of a 6-week-old CNTF$^{-/-}$ mouse. (c) Facial motoneurons of a 6-week-old CNTF$^{-/-}$/LIF$^{-/-}$ mouse. Motoneurons in 6-week-old CNTF$^{-/-}$ mice were not yet reduced in number, but showed reduced cytoplasmic area (b) in comparison to motoneurons of wild-type mice (a). In contrast, severely atrophic motoneurons could already be detected in 6-week-old CNTF$^{-/-}$/LIF$^{-/-}$ mice (c). Scale bar: 40 μm.

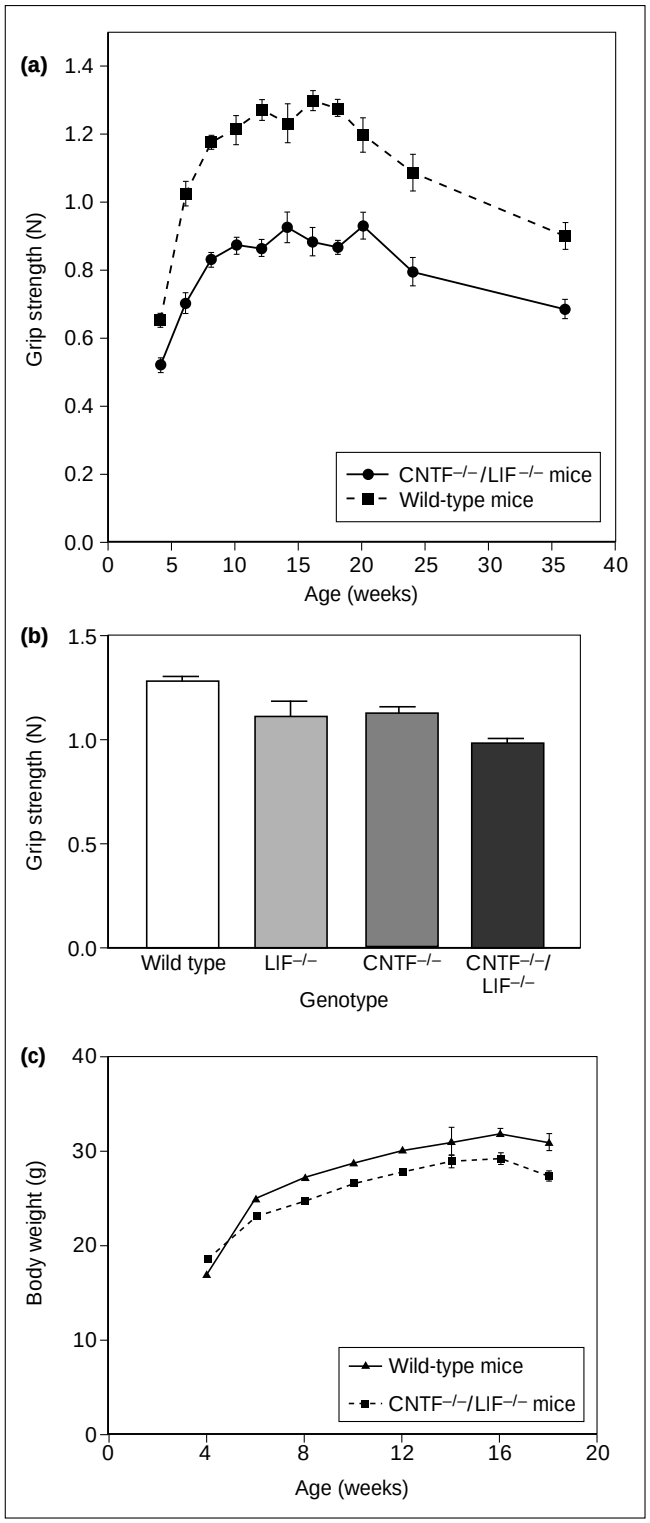
Motor function is more severely impaired in CNTF$^{-/-}$/LIF$^{-/-}$ mice than in CNTF$^{-/-}$ mice

In order to evaluate whether the loss and morphological alterations of motoneurons in CNTF$^{-/-}$/LIF$^{-/-}$ mice were reflected in a reduction of muscle strength, we measured grip strength in these mice at different times after birth. Recent analysis of muscle strength of CNTF$^{-/-}$ mice has revealed that these mice do not develop a significant reduction in muscle strength (measured in Newtons, N) before postnatal week 28 [34]. At that time, muscle strength was reduced by around 10 % compared to wild-type mice. In contrast, mice lacking both CNTF and LIF showed a reduction in muscle strength of about 20 % at 4 weeks, and a reduction of more than 30 % at 18 weeks (Fig. 3a). At this time, average grip strength in mice deficient for both CNTF and LIF was 0.87 ± 0.05 N, compared to 1.10 ± 0.1 N in mice deficient for LIF and 1.28 ± 0.07 N in wild-type mice (Fig. 3b). At stages later than 18 weeks, a similar reduction of grip strength was observed: at 6 months, grip strength in double-knockout mice (n = 9) was 0.79 ± 0.06 N (mean ± SD) compared to 1.09 ± 0.14 N in wild-type mice (n = 6), 0.95 ± 0.07 N in LIF$^{-/-}$ mice (n = 3) and 0.94 ± 0.17 N in CNTF$^{-/-}$ mice (n = 9). At 9–12 months, grip strength in CNTF$^{-/-}$/LIF$^{-/-}$ mice (n = 13) was reduced to 0.67 ± 0.09 N. At this time the grip strength of wild-type mice (n = 11) was 0.90 ± 0.14 N, in LIF$^{-/-}$ mice (n = 2) 0.75 ± 0.11 N and in CNTF$^{-/-}$ mice (n = 4) 0.74 ± 0.11 N. The differences between these groups were statistically significant. The average body weight of 18-week-old CNTF$^{-/-}$/LIF$^{-/-}$ animals was 27 ± 1 g, and wild-type animals weighed 31 ± 2 g at the same age. LIF-deficient animals did not show any reduction of body weight (31 ± 4 g) at this time point, nor was there any evidence for significant differences at any other time point between birth and 9 months of age (data not shown). This shows that the reduced muscle strength observed in CNTF$^{-/-}$/LIF$^{-/-}$ animals is not a non-specific consequence of body weight reduction, but is due to a reduction in motoneuron numbers (and possibly impaired function in residual motoneurons).

The effect of lesion of the facial nerve in 4-week-old CNTF$^{-/-}$ and CNTF$^{-/-}$/LIF$^{-/-}$ mice

Both CNTF and LIF are capable of rescuing motoneurons from massive degeneration after lesion of motor nerves in newborn rats when they are applied locally at the lesion site [30–32]. Motoneurons lose this high vulnerability to lesion-induced degeneration during the 3 postnatal weeks, and lesion of the facial nerve at 4 weeks of age does not result in significant reduction of motoneurons within two

weeks, particularly in mice of the genetic background used in this study. We have proposed that the expression of endogenous CNTF, which reaches maximal levels by the end of the third postnatal week [35], might contribute to the survival of motoneurons after lesion in adult animals compared to newborns.



In order to test this hypothesis, facial nerve transection was carried out in CNTF$^{-/-}$ animals at 4 weeks, when motoneurons have not yet been lost [34]. Motoneuron numbers were determined 2 weeks later in paraffin serial sections of the lesioned and contralateral sides of the brain-stem region that contained the facial nuclei. After facial nerve lesion in CNTF$^{-/-}$ mice, the number of facial motoneurons on the lesioned side was still 90 % of that on the control side (Table 2); the number in the latter was not significantly different from that in wild-type mice. This either indicates that motoneurons did not need CNTF for their survival after lesion at age 4 weeks, or that other factors, such as LIF, compensated for the lack of CNTF. After peripheral nerve lesion, LIF expression is rapidly upregulated within 24 hours, both in the proximal and the distal nerve stump [28]. We therefore also determined motoneuron survival after lesion in CNTF$^{-/-}$/LIF$^{-/-}$ mice. In contrast to CNTF$^{-/-}$ mice, there was a significant loss of motoneurons after lesion at 4 weeks of age. In comparison to the unlesioned side, 34 % of motoneurons were lost, compared to 10 % in CNTF$^{-/-}$ mice. This result indicates that LIF and CNTF cooperate in maintaining motoneuron survival after lesion in 4-week-old mice.

LIF mRNA levels in the sciatic nerves of CNTF$^{-/-}$ mice

Previous studies have indicated that the levels of LIF expression in peripheral nerves of adult rats are very low [28,36]. We have now determined the levels of LIF mRNA in mouse sciatic nerves in 4- and 12-week-old animals. Initial northern blot analysis demonstrated that the levels of LIF mRNA in unlesioned sciatic nerve were so low that a reliable determination was not possible (as reported previously in rat [28]). Therefore, we have used a quantitative detection method based on the polymerase

Figure 3

Forelimb grip strength and body weight of male control and CNTF$^{-/-}$/LIF$^{-/-}$ knockout mice. (a) Comparison of grip strength between wild-type and CNTF$^{-/-}$/LIF$^{-/-}$ mice at different postnatal ages. (b) Comparison of grip strength between 18-week-old male mice of different genotypes. (c) Comparison of body weight of male wild-type and CNTF$^{-/-}$/LIF$^{-/-}$ mice. Grip strength (in Newtons, N) was determined as described previously for CNTF$^{-/-}$ mice [34]. Briefly, mice were allowed to grip a triangular ring of an automated grip strength meter (Columbus Instruments), and then were pulled away until the grip was opened. In contrast to CNTF$^{-/-}$ mice which show only about 10 % reduction in grip strength at age 28 weeks [34], the grip strength of CNTF$^{-/-}$/LIF$^{-/-}$ mice was already reduced by more than 30 % at age 18 weeks. Values shown are means (± SD) from at least six determinations per group. Statistical significance of the differences between groups shown in (b) was tested by one-way ANOVA. The groups were different at $p < 0.0001$. In order to compare the individual groups, the data were further analyzed by Dunnet's multiple comparison test. The differences between wild-type and CNTF$^{-/-}$, wild-type and CNTF$^{-/-}$/LIF$^{-/-}$, and CNTF$^{-/-}$ and CNTF$^{-/-}$/LIF$^{-/-}$ mice were significant at the $P < 0.05$ level; the grip strength measurements in CNTF$^{-/-}$ and LIF$^{-/-}$ mice were not significantly different.

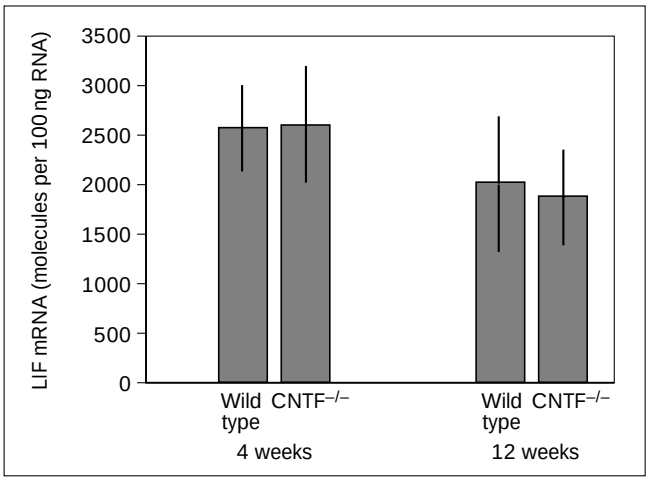
Table 2

Effect of facial nerve lesion on the number of facial motoneurons in CNTF$^{-/-}$ LIF$^{-/-}$ mice.

Genotype	Age	Control side	Side with lesion	Percentage change (%)
Wild-type (n = 5)	6 weeks	2051 ± 101	2374 ± 40	115 ± 5
CNTF$^{-/-}$/LIF$^{+/+}$ (n = 4)	6 weeks	1878 ± 196	1687 ± 217	90 ± 10
CNTF$^{-/-}$/LIF$^{-/-}$ (n = 4)	6 weeks	1677 ± 216	1113 ± 143	66 ± 11

The facial nerve was transected unilaterally at 4 weeks and the mice were analyzed at 6 weeks of age. The differences in the ratios between the lesion side and the control side in the three groups were tested by one-way ANOVA and were found to be significant at the $p < 0.05$ level. Further analysis of the individual groups by Dunnet's multiple comparison test and by Student's t-test showed that the difference between wild-type and CNTF$^{-/-}$ mice was not significant, whereas the difference between wild-type and double-knockout mice was significant at the $p < 0.05$ level.

Figure 4



Quantitative analysis of LIF mRNA expression in the sciatic nerve of CNTF$^{+/+}$ and CNTF$^{-/-}$ mice. Total RNA was reverse-transcribed and amplified by PCR in the presence or absence of a competitor RNA standard that was identical to the LIF sequence apart from a small deletion. Serial dilution of the competitor RNA in the presence of a constant amount of tissue RNA allowed the determination of the amount of LIF mRNA in sciatic nerve. In both CNTF/$^{++}$ and CNTF$^{-/-}$ sciatic nerves, equivalent PCR product yields were found at a concentration of 0.5–2 fg of competitor RNA in sciatic nerves from 4- and 12-week-old mice, which corresponded to about 2 500 LIF mRNA molecules per 100 ng total RNA. Values shown are the mean of at least three determinations in each group; bars represent SD.

chain reaction (PCR). In 100 ng total mRNA isolated from 4-week-old mouse sciatic nerves, 6.2 fg specific LIF mRNA, corresponding to about 2 500 molecules, could be detected. At the age of 12 weeks, lower, but still significant, levels (4.8 fg specific LIF mRNA per 100 ng total mRNA) were detected (Fig. 4). There was no difference between CNTF$^{-/-}$ and wild-type mice. These data indicate that no increase of LIF mRNA expression occurs in Schwann cells at a time when motoneurons degenerated in CNTF$^{-/-}$ animals.

Discussion

In this study, we have compared the consequences of inactivating the CNTF, LIF, and combined CNTF and LIF genes on postnatal survival of motoneurons in mice. We found no reduction in the numbers of motoneuron cells in the facial nuclei of any of these mutant mice at birth. In contrast to CNTF$^{-/-}$ mice, no loss of motoneurons could be detected in LIF$^{-/-}$ animals during postnatal development up to nine months. CNTF$^{-/-}$/LIF$^{-/-}$ mice showed distinct signs of facial motoneuron atrophy and significant functional motor deficits at 6 months of age. Furthermore, a significant reduction of motoneuron numbers was already detectable at 4 weeks of age, at a time when no loss was detectable in CNTF$^{-/-}$ animals [34]. In CNTF$^{-/-}$ animals, lesion of the facial nerve at 4 weeks (when endogenous CNTF levels in Schwann cells have reached maximal levels in control mice) resulted only in small motoneuron losses, which did not reach the level of statistical significance ($p > 0.05$). In contrast, more than 30 % of motoneurons were lost during the two weeks after lesion in CNTF$^{-/-}$/LIF$^{-/-}$ mice. Thus, both CNTF and LIF expression are important for postnatal survival and maintenance of motoneurons.

Both CNTF [12] and LIF [13] are potent survival factors for isolated embryonic motoneurons. These observations have been extended by analysis of the effects of CNTF and LIF on motoneuron survival *in vivo* after lesion of motor nerves in perinatal rats. The results of these studies showed that CNTF [30], as well as LIF [31,32], could prevent death of motoneurons after axotomy. This functional overlap of CNTF and LIF is reflected by the fact that they share gp130 and LIFRβ as signal-transducing receptor components in responsive cells. However, significant differences exist between these two ligands with respect to their specific physiological function for motoneurons. In contrast to LIF, CNTF lacks a hydrophobic leader sequence [35,37] and is not released from synthesizing cells by the conventional secretory pathway. CNTF is expressed at relatively high levels in myelinating Schwann cells, and large quantities of CNTF protein are stored in the cytoplasm of these cells. In unlesioned nerves, the levels of CNTF released seem to be very low, and it is the release of CNTF from Schwann cells that seems to limit its availability to responsive neurons, rather than the expression of CNTF mRNA. The levels of LIF mRNA in unlesioned sciatic nerves of 4- and 12-week-old mice are at least 50 times lower than those of CNTF mRNA. After peripheral nerve lesion, CNTF immunoreactivity is detectable for at least one week at extracellular sites

within the nerve from where it might become available to regenerating motoneurons [25]. This has led to the hypothesis that the cytoplasmic CNTF in myelinating Schwann cells serves as a lesion factor after its release is initiated by the disintegration of the glial cell membrane. Thus CNTF would become available immediately after lesion, whereas LIF, whose basal expression is low but increases after lesion [28], comes into play at later times.

Specific binding of CNTF to its whole receptor complex is mediated by binding to CNTFRα (reviewed in [17,23]), which is linked to the cell membrane via a glycosyl phosphatidyl inositol anchor and which also exists in soluble form in body fluids. LIF does not bind to the CNTFRα receptor and does not displace CNTF from this binding site [38–40]. Interestingly, the concentration of LIF necessary to promote half-maximal survival of isolated rat motoneurons is in a range of 0.1–0.5 ng ml^{-1} [13], about 10 times higher than the concentrations of CNTF supporting half-maximal survival of responsive rat neurons, and about 10–20 times higher than concentrations of LIF necessary for half-maximal biological responses of LIF in the M1 cell line or in hepatoma cells [18,20,41]. In addition, the effect of 10 μg of recombinant rat LIF on facial motoneuron survival is lower than the effect of 5 μg recombinant CNTF after facial nerve transection in newborn rats [31]. This raises the question of whether the putative additional component of the LIF receptor functioning as a high-affinity converter in the M1 cell line and in rat hepatoma cells [42] is expressed in motoneurons. The existence of such a binding protein can be deduced from studies on the retrograde transport of both CNTF and LIF [28,29]. Retrograde transport of both CNTF and LIF is extremely low in unlesioned motoneurons but increases significantly after nerve lesion. Interestingly, the retrograde transport of CNTF in motoneurons can be blocked by a 20-fold excess of LIF [29], whereas a 20-fold excess of CNTF cannot block retrograde transport of LIF. The reason for this striking discrepancy is still unclear but points to the existence of an additional receptor component for LIF in motoneurons, which either cannot bind CNTF or is expressed at such high levels that even 20-fold excess of CNTF cannot effectively compete for the binding to LIF receptors. This demonstrates that the functional CNTF and LIF receptors are different in motoneurons.

The observation that elimination of a gene does not lead to phenotypic alterations in mutant mice but becomes functionally relevant when combined with inactivation of a second, functionally related gene, is not without precedence. For example, inactivation of either MyoD or Myf-5 does not lead to morphological or physiological abnormalities in skeletal muscle development and function [43,44]. However, the expression of Myf-5 is increased 3.5-fold in MyoD-mutant mice, and double inactivation of MyoD and Myf-5 abolishes the formation of skeletal muscle in mutant mice [45]. LIF mRNA is not upregulated in sciatic nerves of CNTF knockout mice at 4 and 12 weeks after birth (Fig. 4). Apparently, regulatory mechanisms (as observed with Myf-5 mRNA in MyoD knockout mice) do not occur in $CNTF^{-/-}$ mice. Nevertheless, a greater reduction in the number of motoneurons is detectable in $CNTF^{-/-}$ $LIF^{-/-}$ mice compared to CNTF mutant mice. This has important consequences for motor function: the loss of muscle strength is significantly greater in $CNTF^{-/-}/LIF^{-/-}$ mice than in $CNTF^{-/-}$ mice. Thus, LIF, although expressed only at low levels in peripheral nerves, contributes to compensatory mechanisms that prevent functional deficits in $CNTF^{-/-}$ mutant mice. Moreover, after axotomy, the presence of LIF in the peripheral nerves of 4-week-old mice seems to protect motoneurons from degeneration to a significant extent.

These observations are also clinically relevant. A mutation of the CNTF gene that leads to an inactive gene product has been discovered both in healthy subjects and in people with neurological disease [5]. This mutation is quite common both in Japan and in the European population (R. Giess and M.S., unpublished observations). About 2 % of the population in Japan and Europe lack functional CNTF expression but do not exhibit any neurological disorders. This indicates that functional deficits, such as muscle paralysis, do not occur, most probably because other gene products, such as LIF, function in a compensatory manner. It would be interesting to determine whether gene defects for LIF exist in humans, and whether combined gene defects of CNTF and LIF could cause motoneuron disease. Such multigenic mechanisms are relevant, because amyotrophic lateral sclerosis (ALS), the most common form of human motoneuron disease, occurs sporadically in more than 90 % of all cases and does not exhibit classical Mendelian inheritance. Thus it might be interesting to know whether combined defects of CNTF and LIF genes, possibly also in combination with other gene defects or epigenetic influences, play a pathogenic role in patients with sporadic ALS.

Conclusions

CNTF and LIF cooperate in the postnatal maintenance of the structure and function of motoneurons. Inactivation of the LIF gene leads neither to a loss of motoneurons nor to a reduction in muscle strength. The inactivation of the CNTF gene alone leads to a 20 % reduction in the number of motoneurons and a borderline reduction in muscle strength. However, the inactivation of both CNTF and LIF genes leads to a more rapid and extensive degeneration of motoneurons (their numbers were reduced by more than 25 %) and to a reduction of muscle strength of more than 30 %. This indicates that degenerative disorders of motoneurons in humans could be caused by similar combinations of gene defects, which individually do not lead to functional defects. The widespread polymorphism

of the human CNTF gene, which results in an inactive gene product, could therefore be a predisposing condition for the development of motoneuron disease if it is combined with other gene defects. The CNTF$^{-/-}$/LIF$^{-/-}$ mouse could therefore be a model for human motoneuron disease of multigenic origin.

Materials and methods

CNTF and LIF knockout mice

Mice with homologous recombination of the CNTF gene [34] were crossbred with LIF$^{-/-}$ mice [46]. Initially, heterozygous mice of each phenotype were crossbred, and the offspring analyzed for the individual genotypes by Southern blot analysis of tail DNA as described [34,46]. Mice exhibiting the individual phenotypes analyzed in this study were derived from animals of this F1 generation.

Facial nerve transection

Control CNTF$^{-/-}$ and CNTF$^{-/-}$/LIF$^{-/-}$ mice (both 4 weeks old) were deeply anaesthetized with ether. The skin behind the right ear was sectioned, and the facial nerve was exposed at the foramen stylomastoideum and transected with fine scissors. The stumps were deflected, and the skin wound was closed using silk (Ethicon 6-0). The effect of the facial nerve transection was detectable by unilateral absence of whisker movement. Fourteen days later, the mice were killed, perfused and brain stems were processed as described below.

Histological analysis

Mice were perfused with 4 % paraformaldehyde. The brain stem was dissected, embedded in paraffin and serial sections (7 μm) were prepared and processed as described [34]. After Nissl staining, the number of neurons exhibiting clear Nissl structure and containing a clearly detectable nucleolus was counted in every fifth section. This number was corrected for double-counting of split nucleoli as described previously [34].

Determination of LIF mRNA

Total RNA was isolated from sciatic nerves of 4- and 12-week-old mice according to Chomczynski and Sacchi [47] using Trizol reagent (Gibco). The yield of RNA was quantified by UV spectrophotometry. For reverse transcription a LIF-specific primer (5′–ACGGTACTT-GTTGCACAGAC) was annealed at 70 °C to a 100 ng aliquot of the RNA and the reaction was started by the addition of Superscript II reverse transcriptase (Gibco) according to the manufacturer´s instructions. Similarly, tissue RNA was added individually to tubes containing a serially decreasing amount of an RNA reference standard. The RNA standard was identical to the LIF sequence, except for an internal deletion of 81 bases, and functions as a competitor in the subsequent PCR. It was synthesized by *in vitro* transcription with T7 RNA polymerase from a plasmid DNA template in which an 81 bp *Nco*I–*Sma*I fragment in exon 3 of the LIF encoding sequence had been deleted. An aliquot of the resulting cDNA was amplified in a standard PCR using the forward primer 5′–ACCCTGTAAATGCCACCTG (located in exon 2) and the reverse primer 5′–CAACGACCATTGCTGAGGAGG (located in exon 3 of the LIF gene). Thus the LIF cDNA transcribed from tissue mRNA results in the amplification of a 378 bp fragment, whereas the competitor RNA is 297 bp long. Because the concentration of the tissue RNA was kept constant in each tube, and the concentration of the competitor was varied, the resulting PCR products were at equimolar concentrations when both RNA hybridization signals were of equivalent density in autoradiograms hybridized with the specific LIF cDNA probe.

Acknowledgements

We thank W. Komp and H. Döppler for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft, Germany, Grant To 61/8-1.

References

1. Thoenen H, Hughes RA, Sendtner M: Trophic support of motoneurons: physiological, pathophysiological, and therapeutic implications. *Exp Neurol* 1993, 124:47–55.
2. Li L, Oppenheim RW, Lei M, Houenou LJ: Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. *J Neurobiol* 1994, 25:759–766.
3. Yan Q, Elliott JL, Matheson C, Sun J, Zhang L, Mu X, *et al.*: Influences of neurotrophins on mammalian motoneurons ***in vivo***. *J Neurobiol* 1993, 24:1555–1577.
4. Griesbeck O, Parsadanian AS, Sendtner M, Thoenen H: Expression of neurotrophins in skeletal muscle: quantitative comparison and significance for motoneuron survival and maintenance of function. *J Neurosci Res* 1995, 42:21–33.
5. Takahashi R, Yokoji H, Misawa H, Hayashi M, Hu J, Deguchi T: A null mutation in the human CNTF gene is not causally related to neurological diseases. *Nat Genet* 1994, 7:79–84.
6. Bazan JF: Neuropoietic cytokines in the hematopoietic fold. *Neuron* 1991, 7:197–208.
7. McDonald NQ, Panayotatos N, Hendrickson WA: Crystal structure of dimeric human ciliary neurotrophic factor determined by MAD phasing. *EMBO J* 1995, 14:2689–2699.
8. Robinson RC, Grey LM, Staunton D, Vankelecom H, Vernallis AB, Moreau JF, *et al.*: The crystal structure and biological function of leukemia inhibitory factor: implications for receptor binding. *Cell* 1994, 77:1101–1116.
9. Kishimoto T, Taga T, Akira S: Cytokine signal transduction. *Cell* 1994, 76:253–262.
10. Baumann H, Schendel P: Interleukin-11 regulates the hepatic expression of the same plasma protein genes as interleukin-6. *J Biol Chem* 1991, 266:20424–20427.
11. Pennica D, King KL, Shaw KJ, Luis E, Rullamas J, Luoh SM, *et al.*: Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc Natl Acad Sci USA* 1995, 92:1142–1146.
12. Arakawa Y, Sendtner M, Thoenen H: Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. *J Neurosci* 1990, 10:3507–3515.
13. Martinou JC, Martinou I, Kato A: Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons ***in vitro***. *Neuron* 1992, 8:737–744.
14. Rao MS, Landis SC: Cell interactions that determine sympathetic neuron transmitter phenotype and the neurokines that mediate them. *J Neurobiol* 1993, 24:215–232.
15. Schooltink H, Stoyan T, Roeb E, Heinrich PC, Rosejohn S: Ciliary neurotrophic factor induces acute-phase protein expression in hepatocytes. *FEBS Lett* 1992, 314:280–284.
16. Dittrich F, Thoenen H, Sendtner M: Ciliary neurotrophic factor: pharmacokinetics and acute phase response. *Ann Neurol* 1994, 35:151–163.
17. Stahl N, Yancopoulos GD: The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. *J Neurobiol* 1994, 25:1454–1466.
18. Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J, *et al.*: The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* 1992, 255:1434–1437.
19. Gearing DP, Thut CJ, Van de Bos T, Gimpel SD, Delaney PB, King J, *et al.*: Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. *EMBO J* 1991, 10:2839–2848.
20. Baumann H, Ziegler SF, Mosley B, Morella KK, Pajovic S, Gearing DP: Reconstitution of the response to leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor in hepatoma cells. *J Biol Chem* 1993, 268:8414–8417.
21. Davis S, Aldrich TH, Ip NY, Stahl N, Scherer S, Farruggella T, *et al.*: Released form of cntf receptor-alpha component as a soluble mediator of cntf responses. *Science* 1993, 259:1736–1739.
22. Ip NY, Nye S, Boulton T, Davis S, Taga T, Li Y, *et al.*: CNTF and LIF act on neuronal cells via shared signalling pathways that involve the IL-6 signal transducing receptor component gp130. *Cell* 1992, 69:1121–1132.
23. Stahl N, Yancopoulos GD: The alphas, betas, and kinases of cytokine receptor complexes. *Cell* 1993, 74:587–590.
24. Ip NY, McClain J, Barrezueta NX, Aldrich TH, Pan L, Li YP, *et al.*: The alpha component of the cntf receptor is required for signaling and defines potential cntf targets in the adult and during

development. *Neuron* 1993, 10:89–102.
25. Sendtner M, Stöckli KA, Thoenen H: Synthesis and location of ciliary neurotrophic factor in the rat sciatic nerve of the adult rat after lesion and during regeneration. *J Cell Biol* 1992, 118:139–148.
26. Friedman B, Scherer SS, Rudge JS, Helgren M, Morrisey D, McClain J, *et al.*: Regulation of ciliary neurotrophic factor expression in myelin-related schwann cells ***in vivo***. *Neuron* 1992, 9:295–305.
27. McNeil PL, Ito S: Molecular traffic through plasma membrane disruptions of cells ***in vivo***. *J Cell Sci* 1990, 96:549–556.
28. Curtis R, Scherer SS, Somogyi R, Adryan KM, Ip NY, Zhu Y, *et al.*: Retrograde axonal transport of LIF is increased by peripheral nerve injury: Correlation with increased LIF expression in distal nerve. *Neuron* 1994, 12:191–204.
29. Curtis R, Adryan KM, Zhu Y, Harkness PJ, Lindsay RM, DiStefano PS: Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. *Nature* 1993, 365:253–255.
30. Sendtner M, Kreutzberg GW, Thoenen H: Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. *Nature* 1990, 345:440–441.
31. Hughes RA, Sendtner M, Thoenen H: Members of several gene families influence survival of rat motoneurons ***in vitro*** and ***in vivo***. *J Neurosci Res* 1993, 36:663–671.
32. Cheema SS, Richards LJ, Murphy M, Bartlett PF: Leukaemia inhibitory factor rescues motoneurones from axotomy-induced cell death. *Neuroreport* 1994, 5:989–992.
33. Rao MS, Sun Y, Escary JL, Perreau J, Tresser S, Patterson PH, *et al.*: Leukemia inhibitory factor mediates an injury response but not a target-directed developmental transmitter switch in sympathetic neurons. *Neuron* 1993, 11:1175–1185.
34. Masu Y, Wolf E, Holtmann B, Sendtner M, Brem G, Thoenen H: Disruption of the CNTF gene results in motor neuron degeneration. *Nature* 1993, 365:27–32.
35. Stöckli KA, Lottspeich F, Sendtner M, Masiakowski P, Carroll P, Götz R, *et al.*: Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. *Nature* 1989, 342:920–923.
36. Patterson PH, Fann MJ: Further studies on the distribution of CDF/LIF mRNA. In *Ciba Foundation Symposium 167*. New York:Wiley; 1992:125–140.
37. Lin L-F, Mismer D, Lile JD, Armes LG, Butler ET III, Vannice JL, *et al.*: Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). *Science* 1989, 246:1023–1025.
38. Wong V, Pearsall D, Arriaga R, Ip NY, Stahl N, Lindsay RM: Binding characteristics of ciliary neurotrophic factor to sympathetic neurons and neuronal cell lines. *J Biol Chem* 1995, 270:313–318.
39. Huber J, Dittrich F, Phelan P: Characterization of high-affinity and low-affinity receptors for ciliary neurotrophic factor. *Eur J Biochem* 1993, 218:1031–1039.
40. Stahl N, Davis S, Wong V, Taga T, Kishimoto T, Ip NY, *et al.*: Cross-linking identifies leukemia inhibitory factor-binding protein as a ciliary neurotrophic factor receptor component. *J Biol Chem* 1993, 268:7628–7631.
41. Gearing DP, Bruce AG: Oncostatin M binds the high-affinity leukemia inhibitory factor receptor. *New Biol* 1992, 4:61–65.
42. Gearing DP: The leukemia inhibitory factor and its receptor. *Adv Immunol* 1993, 53:31–58.
43. Rudnicki MA, Braun T, Hinuma S, Jaenisch R: Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell* 1992, 71:383–390.
44. Braun T, Rudnicki MA, Arnold HH, Jaenisch R: Targeted inactivation of the muscle regulatory gene Myf-5 results in abnormal rib development and perinatal death. *Cell* 1992, 71:369–382.
45. Rudnicki MA, Schnegelsberg PN, Stead RH, Braun T, Arnold HH, Jaenisch R: MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* 1993, 75:1351–1359.
46. Escary J-L, Perreau J, Duménil D, Ezine S, Brûlet P: Leukaemia inhibitory factor is necessary for maintenance of haematopoietic stem cells and thymocyte stimulation. *Nature* 1993, 363:361–364.
47. Chomczynski P, Sacchi H: Single-step method of RNA isolation by guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987, 162:156–159.